Supplementary Material to

Comparison of the Ct Values for genomic and subgenomic SARS-CoV-2 RNA Reveals Limited Predictive Value for the Presence of Replication Competent Virus

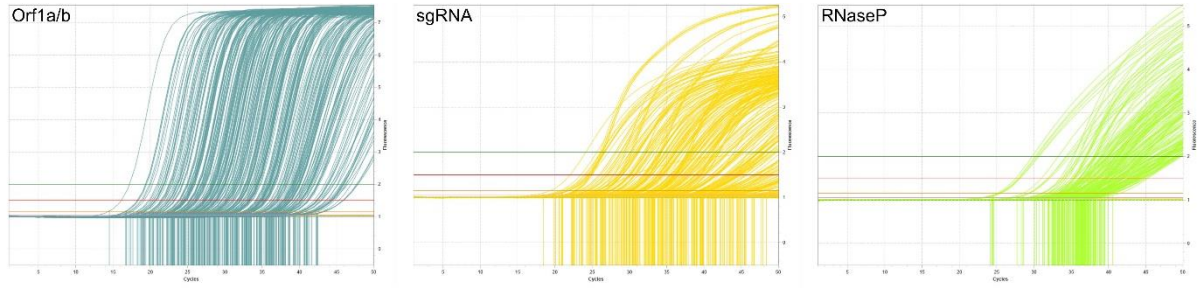
Fabian Roesmann[1], Irene Jakobsche[1], Christiane Pallas[1], Alexander Wilhelm[1], Johanna Raffel[1], Niko Kohmer[1], Tuna Toptan[1], Annemarie Berger[1], Udo Goetsch[2], Sandra Ciesek[1,3,4], Marek Widera[1*]

[1] Institute for Medical Virology, University Hospital Frankfurt, Goethe University Frankfurt am Main, Germany.

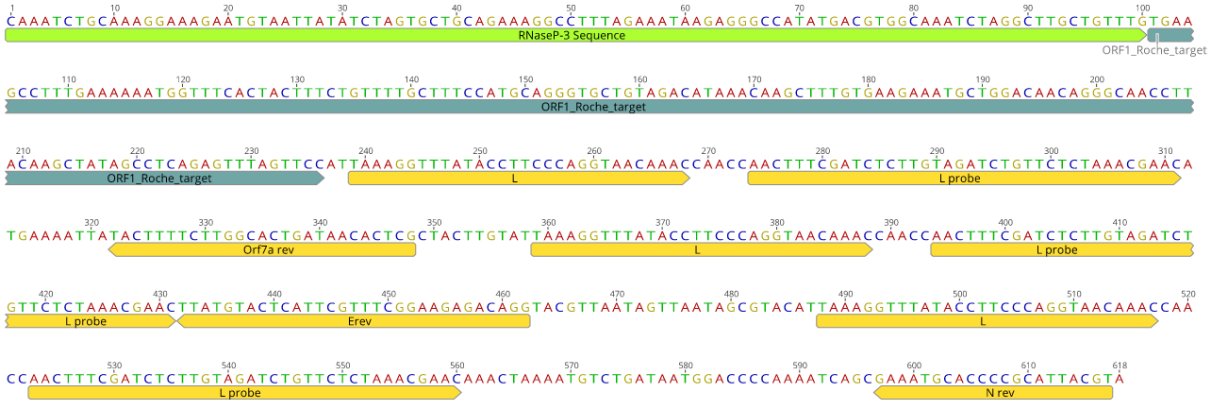
[2] Health Protection Authority, City of Frankfurt, Frankfurt am Main, Germany

[3] German Center for Infection Research, DZIF, Braunschweig, Germany.

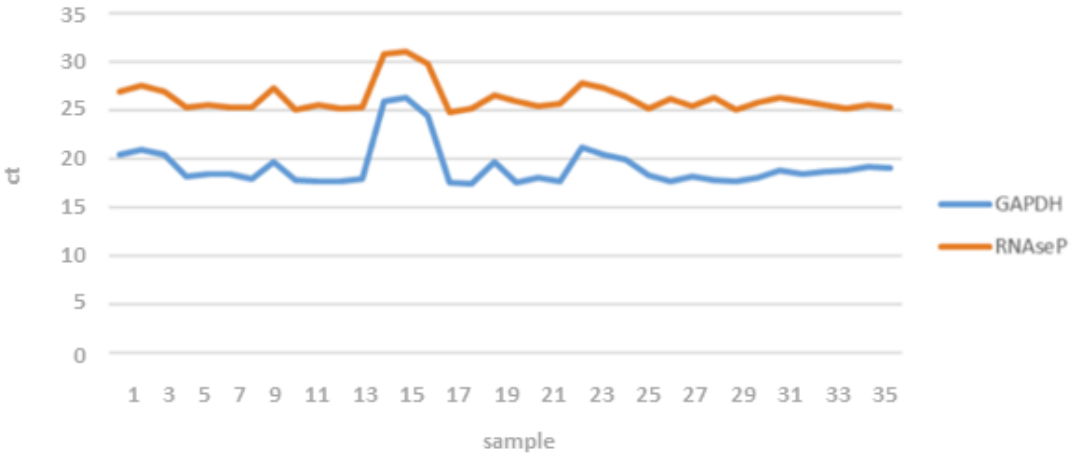
[4] Fraunhofer Institute for Molecular Biology and Applied Ecology (IME), Branch Translational Medicine and Pharmacology, Frankfurt am Main, Germany



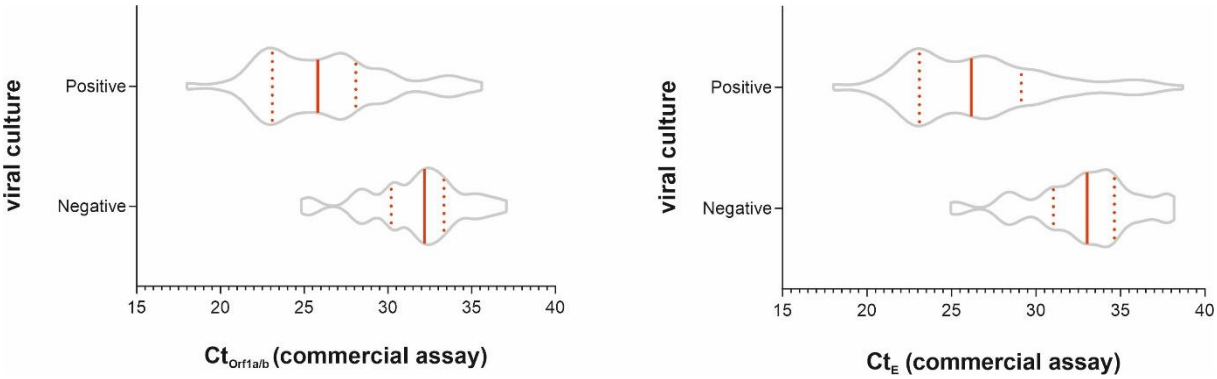
Supplementary Figure 1: Assay performance of the SARS-CoV-2 multiplex PCR assay. The amplification of Orf1a/b (left), the sgRNAs (middle), and a human RNaseP (right) on the cobas omni Utility Channel and the cobas 6800 system (Roche).



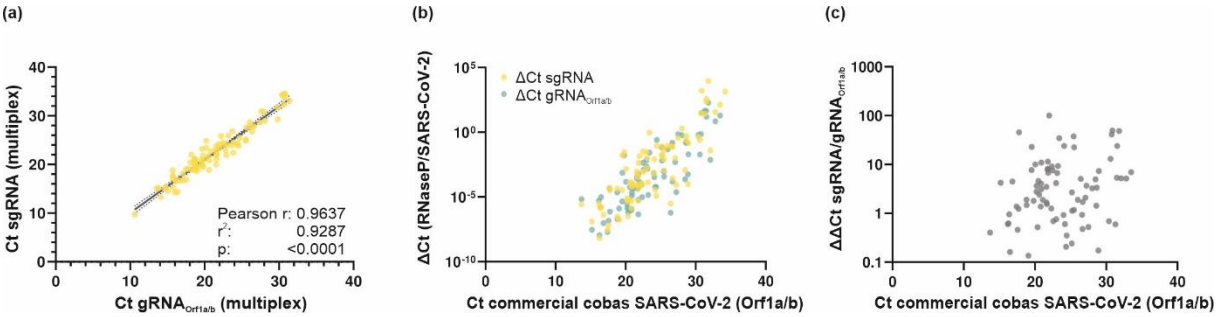
Supplementary Figure 2: Primer and probe binding sites on the RT-qPCR template plasmid. The plasmid contains the spliced RNaseP (loading control) sequence, the Orf1 Roche target (gRNA), and the three most abundant SARS-CoV-2 RNAs, 7a, E, and N (sgRNAs). The binding sites of each primer and probe are annotated.



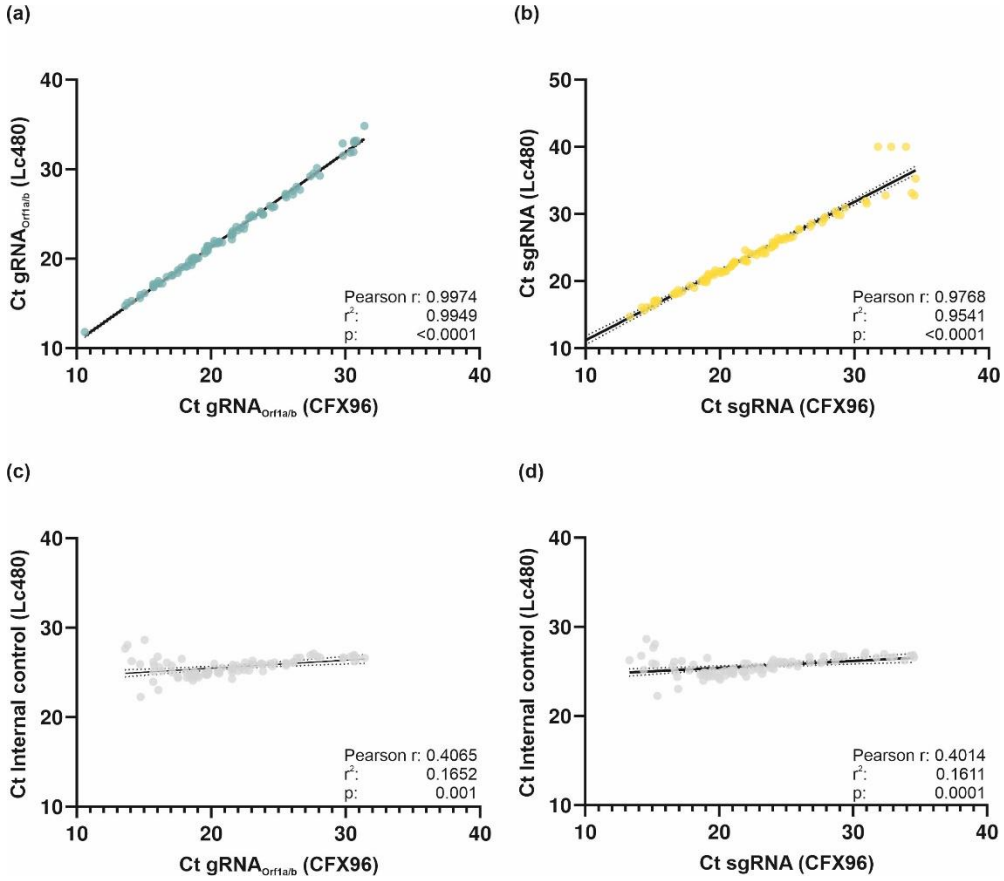
Supplementary Figure 3: Abundance and performance of RNaseP compared to GAPDH as loading control. RNaseP was validated as a loading control by comparing the generated Ct-values against GAPDH in random samples.



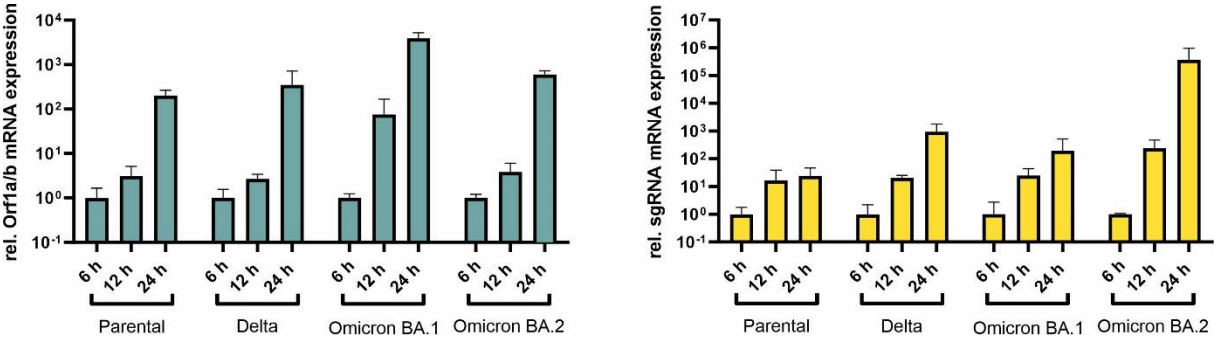
Supplementary Figure 4: Violin plots of the correlation between SARS-CoV-2 Ct-values and viral culture. Ct-values were obtained from the cobas SARS-CoV-2 Test (Roche) targeting SARS-CoV-2 Orf1a/b and E genes. Viral culture assays were performed on Caco2 cells over a period of 7 days after inoculation. If no CPE was visible after 7 days the sample was considered negative. The Ct-values of Orf1 and E separated depending on viral culture. The dotted lines represent the outer quartiles and the dashed lines the median.



Supplementary Figure 5: Inter and intra assay correlation between subgenomic and genomic SARS-CoV-2 RNAs obtained from randomly chosen nasopharyngeal and throat swab samples from routine COVID-19 diagnostics. (a) Ct_{sgRNA} vs. Ct_{gRNA} (Orf1a/b) values were determined using RT-qPCR on the BioRad CFX96 C1000. The solid line represents the best fit regression line, with the shaded area indicating the 95% CI. Pearson index (r), coefficient of correlation (r^2) and significance (p-value) are indicated. **(b)** ΔCt_{sgRNA} or ΔCt_{gRNA} normalized to RNaseP versus Ct_{gRNA} (Orf1a/b) values (commercial cobas 6800 SARS-CoV-2). **(c)** Relative gene expression values calculated by the ΔΔCt-method: $\Delta\Delta ct_{sgRNA\_gRNA}$ (Δct sgRNA – Δct gRNA). The normalized expression of viral RNA (gRNA and sgRNA) was plotted versus the $Ct_{Orf1a/b}$ values (measured by the commercial cobas SARS-CoV-2 test). The solid line represents the best fit regression line, the 95% CI is indicated by the dotted line. Pearson index (r), coefficient of correlation (r2) and significance (p) are indicated if analyzed. Undetected samples were excluded from analyses.



Supplementary Figure 6: Correlation of in-house multiplex assays performed on CFX96 C1000 and LC480. The generated in-house multiplex assay was simultaneously performed on the CFX96 C1000 and the LightCycler 480 System (LC480, Roche). **(a) Ct**$_{gRNA}$ **(**Orf1a/b), and **(b)** Ct$_{sgRNA}$ are plotted against each other. The internal control (IC), solely performed on the LC480, is plotted against **(c)** Ct$_{gRNA}$ (Orf1a/b) or **(d)** Ct$_{sgRNA}$ obtained using the CFX96 machine. For internal control the RNA Process Control Kit (Roche) was used. Undetected samples were excluded from analyses.



Supplementary Figure 7: gRNA and sgRNA expression levels in different SARS-CoV-2 variants.

A549 A+T cells were infected with SARS-CoV-2 variants wildtype (WT), Delta, Omicron BA.1 or BA.2 at an MOI of 0.1. At the indicated time points, cells were washed with PBS, lysed with RLT buffer (Qiagen), and RNA was isolated using the RNeasy 96 QIAcube HT Kit (Qiagen). RT-qPCR was performed on a Bio Rad CFX96 instrument. The expression of gRNA and sgRNA was calculated by ΔCt using GAPDH as a reference gene.

Supplementary Table 1: Lowest and highest obtained Ct-values from the in-house multiplex assay. The minimum and maximum Ct-values of the respective genes are listed. IC = internal control.

	Ct Orf1a/b	***Ct sgRNA***	***Ct RNAseP***	***Ct IC***
Min	14.53	18.45	24.16	31.2
Max	42.48	48.37	49.82	36.38

Supplementary Table 2: Primer and probes used in this study. Name of the primer/probes including position in the host genome within the coding sequence of the indicated gene or RNA leader, respectively. The sequence of each oligonucleotide is given as well as the final concentration (c) used for multiplexing [µM]. Dyes and quenchers (Q) used for each probe are indicated. A universal forward primer within the RNA leader sequence was used for detection of sgRNA E, 7a and N. Nucleotide positions according to SARS-CoV-2 reference genome Wuhan-Hu-1 (NC_045512.2).

Target	Primer/Probe	Position	Sequence	c [µM]	Dye / 3-Q / mid-Q
SARS-CoV-2 gRNA	Orf1a/b fwd	ORF1ab|11980_12007|+	TGAAGCCTTTGAAAAAATGGTTTCACTA	0.6	
	Orf1a/b probe	ORF1ab|12008_12043|+	CTTTCTGTTTTGCTTTCCATGCAGGGTGCTGTAGAC	0.1	56-FAM / 3IABkFQ / ZEN
	Orf1a/b rev	ORF1ab|12115_12087|+	GGAACTAAACTCTGAGGCTATAGCTTGTA	0.6	
SARS-CoV-2 sgRNA	sgRNA fwd	RNA leader |27_56|+	TAAAGGTTTATACCTTCCCAGGTAACAAAC	0.2	
	sgRNA probe	RNA leader [62_99|+	AACTTTCGATCTCTTGTAGATCTGTTCTCTAAACGAAC	0.05	5HEX / 3IABkFQ / ZEN
	sgRNA E rev	CDS E [130_100|+	CCTGTCTCTTCCGAAACGAATGAGTACATAA	0.07	
	sgRNA 7a rev	CDS 7a |136_110|+	CGAGTGTTATCAGTGCCAAGAAAAGAA	0.07	
	sgRNA N rev	CDS N |156_136|+	ACGTAATGCGGGGTGCATTTC	0.07	
Human RNaseP	RNaseP-3 fwd	RNaseP [532_556]+	CAAATCTGCAAAGGAAAGAATGTAA	0.3	
	RNaseP-3 probe	RNaseP [567_597]+	TGCTGCAGAAAGGCCTTTAGAAATAAGAGGG	0.2	5LtC640N / 3IAbRQSp / TAO/
	RNaseP-3 rev	RNaseP [610_631]+	CAAACAGCAAGCCTAGATTTGC	0.3	

Supplementary Table 3: Comparison of Ct-values between established and developed RT-qPCR assays, listed for each day of viral culture positivity. CaCo2 cells were inoculated with SARS-CoV-2 spiked PBS, obtained from nasopharyngeal or throat swaps of SARS-CoV-2 positive tested individuals. Ct-values are separated by the days until positive viral culture of the corresponding sample. The Ct-values of gRNA (Orf1a/b) generated by the commercial RT-qPCR assay and the in-house multiplex RT-qPCR, as well as the Cts of the sgRNA from the multiplex approach are listed. A sample was considered negative if no cytopathic effect (CPE) was monitored 7 days after inoculation. Data are shown as median including interquartile range in brackets.

	Ct value			Total no. (%)	
Viral culture	gRNA (Orf1a/b) commercial test	gRNA (Orf1a/b) in-house multiplex	sgRNA (E, 7a, N) in-house multiplex		
1 day	17.6 **(17.6; 17.6)**	18.3 **(18.3; 18.3)**	22.6 **(22.6; 22.6)**	1	**(0.4)**
2 days	24.0 **(21.8; 26.5)**	24.1 **(21.5; 26.8)**	29.3 **(26.0; 33.0)**	44	**(16.5)**
3 days	23.4 **(20.4; 25.8)**	23.8 **(19.6; 27.0)**	28.9 **(24.5; 33.3)**	34	**(12.7)**
4 days	26.4 **(25.6; 29.7)**	26.9 **(25.6; 30.7)**	32.8 **(31.5; 37.2)**	6	**(2.2)**
5 days	24.4 **(23.4; 25.7)**	25.7 **(24.3; 22.4)**	29.6 **(28.0; 31.4)**	22	**(8.2)**
6 days	25.0 **(23.4; 27.3)**	27.3 **(25.1; 23.4)**	30.9 **(28.2; 35.2)**	3	**(1.1)**
>7 days (Negative)	31.7 **(29.7; 33.2)**	33.2 **(34.3; 31.2)**	39.7 **(35.4; 43.5)**	157	**(58.8)**
				267	**(100)**